

MAGE Xp-2: A Member of the MAGE Gene Family Isolated from an Expression Library Using Systemic Lupus Erythematosus Sera

D. K. McCurdy,*¹ L-Q. Tai,[†] J. Nguyen,[†] Z. Wang,[†] H-M. Yang,[†] N. Udar,[†]
F. Naiem,[†] P. Concannon,[‡] and R. A. Gatti[†]

*Department of Pediatric Rheumatology, Children's Hospital of Orange County, Orange, California 92868; [†]Department of Pathology, University of California, Los Angeles, Los Angeles, California 90024 and; [‡]Virginia Mason Research Center, Seattle, Washington

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Two regions of the genome contain members of the MAGE gene family; Xq27-qter and Xp21.3. We isolated a transcript, MAGE Xp-2, by screening a cDNA library from the human epithelial carcinoma cell line, HEP-2, using autoantibodies from patients with systemic lupus erythematosus (SLE). The open reading frame (ORF) of MAGE Xp-2 is entirely contained in exon 4, a signature feature of the MAGE gene family. While MAGE Xp-2 shares genomic homology with MAGE Xp-1, the predicted proteins are quite divergent. Specific primers were designed to reliably distinguish between MAGE Xp-1 and MAGE Xp-2 expression. MAGE Xp-2 is expressed in testis, but not in other normal tissues. It is also expressed strongly in two of seven melanoma cell lines and one of four breast carcinomas. MAGE gene expression may be important not only for tumor recognition and cancer therapy, but, because it is the apparent target of autoantibodies in SLE sera, it may also play a role in autoimmune diseases. © 1998 Academic Press

Key Words: MAGE genes; DAM genes; chromosome Xp; systemic lupus erythematosus; epithelial carcinoma; breast cancer; melanoma.

The MAGE genes are a family of genes that have the potential to code for antigens that act as targets for specific antitumor cytotoxic T lymphocyte (CTL) responses. MAGE-1, first cloned in 1991 (1), was isolated from a melanoma cell line. Lymphocytes from the melanoma patient produced CD8+ CTL clones

that lysed the MAGE-1-expressing autologous tumor cell line. Subsequently, MAGE-1 was found to be expressed in tumors of various histological types such as melanomas and lung and breast carcinomas (2,3).

In melanoma cell lines, the MAGE-1 gene product is recognized by CTLs in combination with HLA Class I surface antigens (4). The target for the CTL recognition is a nonapeptide presented by HLA-A1 combining to form the antigen, MZ2-E. By probing cosmid libraries with MAGE-1 sequence, 11 closely related genes were subsequently identified, revealing that MAGE-1 belongs to a multigene family. Analyzing DNA from hamster-human somatic cell hybrids localized the 12 MAGE genes to the terminal region of the long arm of chromosome X (Xq27-qter) (5). The other MAGE gene products (MAGE 2-12), although structurally similar to MAGE-1, do not contain the nonapeptide and are not recognized by CTLs with specificity for MZ2-E.

Muscатели *et al.* (6) described a gene in the MAGE family that was located on the short arm of the X chromosome at Xp21.3 (herein referred to as MAGE Xp-1). As with the MAGE Xq gene family members, this gene has its open reading frame in the last exon and the protein it encodes appears to be structurally similar to other members of the MAGE family. Dabovic *et al.* (7) recently described two similar MAGE Xp genes, DAM 6 and DAM 10, the names deriving from "dosage-sensitive sex reversal (DSS)/adrenal hypoplasia congenita (AHC) region of the MAGE superfamily." DAM 10 is identical to MAGE Xp-1.

In studies that screened a human epithelial carcinoma cell (HEP-2) cDNA expression library with au-

¹ To whom correspondence should be addressed at Children's Hospital of Orange County, Rheumatology Division, 455 South Main Street, Orange, CA 92868. Fax: (714) 532-8883.

toantibodies from patients with systemic lupus erythematosus (SLE), originally searching for potential autoantigens, we also isolated a MAGE Xp gene (herein referred to as MAGE Xp-2), which is almost identical to DAM 6 (7). We report here on its expression in normal and malignant tissue, as well as on its structural homology to MAGE Xp-1/DAM 10 and to DAM 6. We also consider a potential role for MAGE Xp-2 in the pathogenesis of SLE.

MATERIALS AND METHODS

Source of sera. The Pediatric Rheumatology Division at the Children's Hospital of Orange County (CHOC) follows approximately 80 children with SLE per year. Blood was collected from children who fulfilled the 1982 ACR revised criteria for SLE, and control sera were collected from those without disease, under a protocol approved by the Institutional Review Board at CHOC. Sera were immediately separated, aliquoted, and stored at -80°C . IgG was affinity-purified by acid elution from a protein G column. The eluants were then passed over *E. coli* lysates and β -galactosidase columns at 4°C to be used for screening the protein expression library.

cDNA and ZAP-II expression library of HEP-2. HEP-2, an epithelial laryngeal carcinoma cell line obtained from American Type Culture Collection (Rockville, MD), was grown to confluency and harvested. Total RNA was prepared using the guanidinium thiocyanate-phenol-chloroform extraction method (8). Messenger RNA (mRNA) was isolated using Stratagene Poly(A) Quik mRNA isolation kit (Stratagene, LaJolla, CA). The Stratagene ZAP-II cDNA synthesis kit and the ExAssist/Solar system kit (Stratagene) were used to prepare the HEP-2 cDNA expression library and λ Zap-II plasmids according to the manufacturer's instructions.

Immunoscreening. The fusion proteins from the cDNA library were expressed on a nutrient plate and transferred to nitrocellulose filters blocked with *E. coli*/phage lysate to prevent nonspecific binding. IgG antibodies from two patients' sera were used to probe for fusion proteins as potential autoantigens. The antibodies binding to the fusion proteins were detected by enhanced chemiluminescence (ECL) (Amersham International, Buckinghamshire, England).

Polymerase chain reaction (PCR). Plasmids were extracted and purified using a WIZARD Plus Miniprep DNA purification system (Promega, Madison, WI). DNA excised from the phage was amplified by

the following method: 100–150 ng of DNA was amplified in a volume of 25 μl PCR buffer (10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl_2 , 0.001% (w/v) gelatin), 100 μM dNTP, 0.8 μM primer, 1 unit of *Taq* 2000 DNA polymerase (Perkins Elmer Cetus, Norwalk, CT). The primers and annealing temperatures are described in Table 1. Because of the high degree of homology between the MAGE genes being compared, many other primers were tested and later set aside for lack of specificity. Amplification was performed in a Perkin-Elmer PCR thermal cycler (Perkins Elmer Cetus) for 35 cycles. The PCR products were separated and characterized by agarose gel electrophoresis.

Reverse transcriptase (RT) PCR. Total RNA was extracted from cell cultures using RNEZ (Qiagen, Chatsworth, CA). For cDNA synthesis, total RNA was pretreated with DNase I (BRL/Life Technologies, Gaithersburg, MD) and reverse-transcribed: 2 μg total RNA was incubated at 37°C for 1 h in a 50- μl volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT, 4 mM dNTP, Pd (N)₆, sodium salt 9×10^{-2} M (Pharmacia, Beverly, MA), 2 units oligo(dT) 18 (Biolab, Piscataway, NJ), 38.95 units RNase inhibitor, and 200 units of M-MLV reverse transcriptase (BRL/Life Technologies). After cDNA synthesis, the PCR was amplified using the same conditions as above and the primers and annealing temperatures described in Table 1.

Rapid amplification of the 5' cDNA end. The 5' RACE system was used following the instructions of the supplier (BRL/Life Technologies). Gsp-1 and Gsp-2 were used to synthesize specific cDNA for PCR. The resulting PCR product was cloned into pAMP1 vector (BRL/Life Technologies) and five clones were sequenced.

DNA sequencing. Sequencing was performed on double-stranded DNA. Plasmids were extracted and purified using the WIZARD Plus minipreps DNA purification system (Promega). The PCR product was run on a 1% agarose gel and purified with a QIAquick gel extract kit (Qiagen). Sequencing was performed with a Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, OH) and dsDNA cycle sequencing system (BRL/Life Technologies).

Northern blot electrophoresis. Total RNA was isolated from cell cultures as described above and 10 μg was loaded on a 1% agarose gel with 1X Mops buffer and 0.054%, v/v, of 37% formaldehyde. After electrophoresis, the RNA was transferred to a nylon plus membrane (Qiagen). The blot was prehybrid-

TABLE 1
Mage XP-2 Oligonucleotide Primers

Primer	Sequence 5'-3'	Location (nt)	Annealing temperature (°C)	
LQ-1F	CTG ACT TCC GCT TTG GAG GC	1-20	1 M Na ⁺	72°C
LQ-1R	CCA CCC CCA GAA ACA GAA GAG GAA CA	222-247	10 mM Na ⁺	51°C
XP-2F	TAG CCA ATC AAT CTC CCA AAG CCA AG	1096-1121	1 M Na ⁺	78°C
XP-2R	TCA CTA TGG GGT CTT T	1453-1468	50 mM Na ⁺	74°C
CLT-1F	CAG GTC CTG AAG CAG AGG AA	186-206		53°C
CLT-2R	GGG GTC TTT CAC ATT TCC AA	1442-1461		58°C
GSP-1R	GGG TGA GAA CAG CAG GTA AA	1122-1141		48°C
GSP-2R	(CuA)4 AGG AGA GCT TGA AGC AC	247-263		48°C
				72°C

ized in 50 μ g salmon sperm DNA (BRL/Life Technologies) and 50 μ g human DNA cot-1 (BRL/Life Technologies) in 2 ml QUIKhyb solution (Stratagene) at 65°C for 1 h and hybridized with a [α -³²P]dATP-labeled probe (Amersham, Arlington Heights, IL) under the same conditions for 1 h. Washing conditions were 2X SSC, 0.1% SDS at 60°C for 20 min, 0.5X SSC and 0.1% SDS at 55°C for 10 min, and 0.1X SSC and 0.1% SDS at 50°C for 10 min. A multiple tissue northern (MTN) blot from Clontech (Palo Alto, CA) was hybridized as recommended by the manufacturer. Breast cancer cell lines were obtained from ATCC. Melanoma cell lines were obtained from L. Butterfield, J. Fountain, and H. Vinters.

Southern blot analysis. Genomic DNA was isolated using a DNA isolation kit (GENTRA, Minneapolis, MN), digested, run on the 1% agarose gel, and transferred by standard methods. It was prehybridized and hybridized at 65°C for 1 and 2 h, respectively, in 100 μ g salmon sperm DNA (BRL/Life Technologies) and 100 μ g human DNA cot-1 (BRL/Life Technologies) in 2 ml QUIKhyb solutions. Probes were labeled with a [α -³²P]dCTP in a random reaction. Washing conditions were 2X SSC, 0.1% SDS at 65°C for 20 min, 0.5X SSC and 0.1% SDS at 65°C for 20 min, and 0.1X SSC and 0.1% SDS at 65°C for 20 mins. The washes were repeated twice.

Somatic cell hybrids for chromosomal localization. The Stanford G3 panel of radiation hybrid cell lines (Research Genetics, Huntsville, AL) was screened by PCR with the primers CTL-1F, CTL-2R, and GSP-1R (Table 1). PCR reactions included 25 ng of hybrid DNA, 0.2 μ M of each primer, 0.2 mM nucleotide triphosphates, 0.3 mM spermidine, 10 μ g bovine serum albumin, and reaction buffer supplied by the manufacturer (Gibco BRL) in a total reaction volume of

25 μ l. PCR products were separated on agarose gels and each hybrid was scored as positive, negative, or undetermined for MAGE Xp-2 sequence. Results of this analysis were forwarded to the RHServer at Stanford University (rhsever@shgc.stanford.edu) for two-point linkage mapping.

Homology search. Sequence alignments of DNA and protein sequences were performed with the NCBI computer program (National Center for Information, Bethesda, MD). The computer search for homology in GenBank was done with the BLAST programs using the NCBI server.

RESULTS

Isolation of cDNA from a human epithelial carcinoma cell line (HEp-2) expression library screened with IgG from SLE patients. To isolate genes of potential autoantigens in patients with SLE, we screened a cDNA expression library made from the human epithelial carcinoma cell line, HEp-2, with two affinity-purified sera from pediatric patients with high titer antinuclear antibodies (ANA) and lupus nephritis. The HEp-2 cell line was selected for making an expression library because it has been used by most clinical laboratories as the standard cell for detecting ANA. In preliminary studies sera from 40 pediatric patients with SLE were screened for antinuclear antibodies using immunofluorescence against a panel of peripheral blood mononuclear cells (PBM) from patients with acute lymphoblastic leukemia (ALL) and acute myelocytic leukemia (AML) (9). As expected, all of the samples were positive on HEp-2 cells in the standard assay for ANA. The sera were also positive on 70% of ALL

cells and 63% of AML cells, respectively, showing either a reticular or a speckled pattern (9).

Two sera with high-titer ANA antibodies expressing both the speckled and the reticular patterns were selected for all further studies. IgG was affinity-purified from each by eluting from protein G columns. The eluants were passed over *E. coli* lysate and β -galactosidase columns, diluted 1/500, and used to screen a HEP-2 expression library. Seventeen cDNA clones were independently isolated; after we eliminated clones containing ribosomal cDNA, vector, or repeat sequences, seven clones reacted with both serum IgGs. The seven clones were further analyzed by PCR amplification and restriction enzyme mapping, using *Xho*I and *Eco*R1. Sizes ranged from 0.5 to 3.5 kb. Southern blots confirmed that each clone was unique, with no cross-hybridization between the selected clones (data not shown). The seven clones were sequenced and one, clone 7, showed homology with genes in the MAGE superfamily, but was not identical to any previously described genes at that time. Characterization of the other six clones is in progress.

Sequence analysis of clone 7 and homology with the MAGE superfamily. Figure 1 shows the 1608 nucleotide sequence (GBD Accession Number AF015766) and 319 deduced amino acid sequence of clone 7 (herein referred to as MAGE Xp-2). Rapid amplification of 5' cDNA ends (5' RACE) was used to extend the 5' sequence of clone 7 by 79 nucleotides. A significant similarity of organization was found between the clone 7 cDNA sequence and the previously described genes in the MAGE superfamily. Although the sequence of DAM 6 was not found in the GenBank program, we subsequently noted that our MAGE Xp-2 sequence was nearly identical to the ORF of the published sequence of DAM 6 (7). We also found approximately 77% nucleotide similarity between MAGE Xp-2 and MAGE Xp-1.

Sequence comparison of MAGE Xp-2 with MAGE Xp-1, and DAM 6. Figure 2 shows a sequence alignment of MAGE Xp-1 and MAGE Xp-2. MAGE Xp-1 (6) and MAGE Xp-2 have initiation sites at positions 324 and 102, respectively, in a segment of almost complete identity. MAGE Xp-1 has a termination codon at position 1368. MAGE Xp-2, in contrast, terminates transcription at position 1062, 88 nucleotides upstream of the MAGE Xp-1 site, although MAGE Xp-2 retains a second stop codon at this site as well. Thus, the MAGE Xp-1 ORF is slightly longer. It is of further interest that by a comparison of the sequences of MAGE Xp-1 and MAGE Xp-2,

MAGE Xp-2 appears to have (1) a single serine of MAGE Xp-1 replaced with two cysteines at residues 40 and 41, and (2) one extra alanine at residue 72, creating a string of six alanines. Beginning at about residue 74, the amino acid sequence diverges considerably. Despite this, the histidine at position 88 of MAGE Xp-2 appears to be added to an otherwise homologous region. At position 1253 of MAGE Xp-1, an in-frame deletion of GAG results in the deletion of a glutamic acid from MAGE Xp-2 (Fig. 1, see asterisk).

The ORFs of Xp-2 and DAM 6 are identical except for a T > C transition at position 407 which does not change the amino acid. In the 3' untranslated region, we noted nucleotide changes at three sites: a CT > TC at positions 1087 and 1088, a C > A at position 1393, and a G > T at position 1494. These differences may represent polymorphisms.

We detected a complete divergence between MAGE Xp-2 and MAGE Xp-1 of genomic sequence at the 5' end of exon 3. This sequence was not described by Dabovic *et al.* for DAM 6 (7). Attempts to PCR amplify with reverse primers designed from this unique region of MAGE Xp-2 and forward primers from MAGE Xp-1 exons 1 and 2 were unsuccessful, even when the long-range PCR could be extended to 10 kb for controls. This implies that MAGE Xp-2 does not have upstream exons that are highly homologous to those of MAGE Xp-1.

The exon-intron boundaries of MAGE Xp-2. The MAGE gene superfamily appears to have a conserved structure in all 14 genes described, characterized by the presence of two to four exons, with the ORF in the last exon. To determine exon-intron boundaries and intron sizes, PCR amplification products from cDNA or genomic DNA (gDNA) were compared and sequenced. Figure 3 summarizes these relationships. Using MAGE Xp-2-specific primers spanning exons 3 and 4, an intron of approximately 3 kb was identified in gDNA; this was similar to the intron identified in DAM 6 and MAGE Xp-1/DAM 10. However, we found that MAGE Xp-2 cDNA lacked 55 nucleotides just upstream of the 5' end of exon 4 (see Fig. 2 also), suggesting that the splice site indicated by Muscatelli *et al.* at MAGE Xp-1 positions 263–264 (Fig. 2) is most likely at positions 261–262.

Chromosomal location of MAGE Xp-2. To determine the chromosomal location of MAGE Xp-2 gene, primers specific for the sequences CTL-1F, CTL-2R, and GSP-1R (Table 1) were used to screen the Stanford G3 radiation hybrid cell panel. Results from

nt																			aa
1	TTT	GGA	GGC	GAG	GAC	CCG	AGC	GAG	TGT	AGG	GGG	TGC	CT	GAC	TTC	CGC			
12	GCC	AGG	GGT	GTA	TTC	TCA	GGA	CTG	GTC	GGC	AGT	CAA	GCC	ATC	TGG	TCA			
60															<u>ATG</u>	CCT			
															M	P			2
108	CGT	GGT	CAG	AAG	AGT	AAG	CTC	CGT	GCC	CGT	GAG	AAA	CGC	CGC	AAG	GCC			18
	R	G	Q	K	S	K	L	R	A	R	E	K	R	R	K	A			
156	CGA	GAT	GAG	ACC	CGG	GGT	CTC	AAT	GTT	CCT	CAG	GTC	ACT	GAA	GCA	GAG			
	R	D	E	T	R	G	L	N	V	P	Q	V	T	E	A	E			34
204	GAA	GAA	GAG	GCC	CCC	TGC	<u>TGT</u>	TCC	TCT	TCT	GTT	TCT	GGG	GGT	GCT	GCT			
	E	E	E	A	P	C	C	S	S	S	V	S	G	G	A	A			50
252	TCA	AGC	TCT	CCT	GCT	GCT	GGC	ATT	CCC	CAG	AAG	CCT	CAG	AGA	GCC	CCA			
	S	S	S	P	A	A	G	I	P	Q	K	P	Q	R	A	P			66
300	ACC	ACT	GCC	GCT	GCT	<u>GCA</u>	GCT	GCG	GGT	GTT	TCA	TCC	ACA	AAA	TCT	AAA			
	T	T	A	A	A	A	A	G	V	S	S	T	T	K	S	K			82
348	AAA	GGT	GCC	AAG	AGC	<u>CAC</u>	CAA	GGT	GAG	AAA	AAT	GCA	AGT	TCC	TCC	CAG			
	K	G	A	K	S	H	Q	G	E	K	N	A	S	S	S	Q			96
396	GCC	TCA	ACA	TCT	ACT	AAG	AGC	CCA	AGC	GAA	GAT	CCT	CTA	ACC	AGG	AAG			
	A	S	T	S	T	K	S	P	S	E	D	P	L	T	R	K			114
444	TCA	GGG	TCG	TTG	GTG	CAG	TTT	CTG	TTG	TAC	AAG	TAT	AAA	ATA	AAA	AAG			
	S	G	S	L	V	Q	F	L	L	Y	K	Y	K	I	K	K			130
492	TCC	GTT	ACA	AAG	GGA	GAA	ATG	CTG	AAA	ATT	GTT	GGC	AAA	AGG	TTC	AGG			
	S	V	T	K	G	E	M	L	K	I	V	G	K	R	F	R			146
540	GAG	CAC	TTC	CCT	GAG	ATC	CTC	AAG	AAA	GCC	TCT	GAG	GGC	CTC	AGT	GTT			
	E	H	F	P	E	I	L	K	A	S	A	S	G	L	S	V			162
588	GTC	TTT	GGC	CTT	GAG	CTG	AAT	AAA	GTC	AAC	CCC	AAC	GGC	CAC	ACT	TAC			
	V	F	G	L	E	L	N	K	V	N	P	N	G	H	T	Y			178
636	ACC	TTC	ATC	GAC	AAG	GTA	GAC	CTC	ACT	GAT	GAG	GAA	TCC	CTG	CTC	AGT			
	T	F	I	D	K	V	D	L	T	D	E	E	S	L	L	S			194
684	TCC	TGG	GAC	TTT	CCC	AGG	AGA	AAG	CTT	CTG	ATG	CCT	CTC	CTG	GGT	GTG			
	S	W	D	F	P	R	R	K	L	L	M	P	L	L	G	V			210
732	ATC	TTC	TTA	AAT	GGC	AAC	TCA	GCT	ACT	GAG	GAA	GAG	ATC	TGG	GAA	TTC			
	I	F	L	N	G	N	S	A	T	E	E	E	I	W	E	F			226
780	CTG	AAT	ATG	TTG	GGA	GTC	TAT	GAT	GGA	GAG	GAG	CAC	TCA	GTC	TTT	GGG			
	L	N	M	L	G	V	Y	D	G	E	E	H	S	V	F	G			242
828	GAA	CCC	TGG	AAG	CTC	ATC	ACC	AAA	GAT	CTG	GTG	CAG	GAA	AAA	TAT	CTG			
	E	P	W	K	L	I	T	K	D	L	V	Q	E	K	Y	L			258
876	GAG	TAC	AAG	CAG	GTG	CCC	AGC	AGT	GAT	CCC	CCA	CGC	TTT	CAA	TTC	CTG			
	E	Y	K	Q	V	P	S	S	D	P	P	R	F	Q	F	L			274
926	TGG	GGT	CCG	AGA	GCC	TAT	GCT	GAA	ACC	AGC	AAG	ATG	AAA	GTC	CTG	GAG			
	W	G	P	R	A	Y	A	E	T	S	K	M	K	V	L	E			290
976	TTT	TTG	GCC	AAG	GTA	AAT	GGT	ACC	ACC	CCC	TGT	GCC	TTC	CCA	ACC	CAT			
	F	L	A	K	V	N	G	T	T	P	C	A	F	P	T	H			306
1020	TAC	GAA	GAA	GCT	TTG	AAA	GAT	GAA	GAG	AAA	GCC	GGA	GTC	<u>TGA</u>	GCC	AGA			
	Y	E	E	A	L	K	D*	E	E	K	A	G	V						319
1068	GTT	GTA	GCC	AGG	CCT	TGC	ACT	ACT	GCC	ATA	GCC	AAT	CAA	TCT	CCC	AAA			
1116	CCC	AAG	TTT	ACC	TGC	TGT	TCT	CAC	CCC	CAA	TGA	GGT	CTT	AGG	CAG	ATT			
1164	CTT	TAC	TTT	GTA	ATT	CAA	AAG	GCC	TGT	TAA	CCT	TTG	TTC	TTG	TTA	TGC			
1212	ATG	AAT	AAC	TTG	TTG	ACT	TTT	TTT	TCT	CTT	TTT	CAA	CTA	GTG	TTT				
1260	CAA	CAG	GTT	TAT	TTA	GAT	TCA	GAA	TGT	AAA	TTT	ACA	AAT	GAT	ATA	GAT			
1308	CAC	CCT	GTT	ATT	GCT	GTT	TTT	CAG	GGA	CAG	TAG	AAA	GTG	TTT	TGT	TTT			
1356	TTG	AGT	GAA	ACA	ACT	TAT	TAA	TAA	AAA	TCC	TTA	AAT	CTC	TTT	TGT	AAT			
1404	CCA	GGA	CAA	GAA	AAT	GTG	GCA	TTA	GAA	TAG	AAA	TAT	CTT	TGG	AAA	TGT			
1452	GAA	AGA	CCC	CAT	AGT	GAA	ATA	TTT	GGG	ATC	AGA	AGC	CAG	AGG	GGT	AAA			
1500	AGT	GGT	CAA	TTC	TTG	GTT	TAC	TTC	ATT	TAA	TCT	TTC	TTT	TCA	TAA	AGA			
1548	TAC	ATA	CCT	GGA	TTT	GTT	TAT	GTT	ATT	CAA	GAA	TGT	GTG	AGA	AAT	TAA			
1596	ACC	ATA	GTT	AGT	T														

FIG. 1. cDNA sequence and deduced amino acid sequence of MAGE Xp-2. The predicted start of translation is at nucleotide 102, in exon 4, with the first in-frame stop codon (TGA) terminating transcription at nucleotide 1062, yielding an open reading frame (ORF) of 960 bp that encodes a predicted protein of 319 amino acids. The start and stop codons are bold and underlined. Underlined codons at nucleotides 222, 315, and 362 indicate residues inserted into MAGE Xp-2. The asterisk after amino acid 313 indicates a deleted glutamic acid in MAGE Xp-2 when compared with MAGE Xp-1.

this analysis were used to query the RHServer at Stanford University for two-point linkage mapping. Significant evidence of linkage ($\text{lod} > 6$) was detected between MAGE Xp-2 and multiple markers on chromosome Xp.

Southern blot analysis of genomic DNA. DNA was digested with *Eco*R1, *Hind*III, and *Bam*HI and blotted. It was first probed with PCR-amplified, ^{32}P -labeled MAGE Xp-1 and then with a similar MAGE Xp-2 product. With each restriction enzyme, there were clear differences in the bands detected when

the blot was probed with MAGE Xp-1 or MAGE Xp-2 (Fig. 4). MAGE Xp-1 hybridized to bands of 8.0, 7.8, and 4.0 kb. MAGE Xp-2 detected three new and distinct bands at 10.0, 6.0, and 4.9 kb.

Expression pattern of MAGE Xp-2. A mRNA transcript of 1.7 kb was detected only in human adult testis and not in normal spleen, thymus, prostate, ovary, small intestine, colon, or peripheral blood lymphocytes of a commercially prepared mRNA tissue blot (Fig. 5). A subsequent Northern blot using RNA from the HEp-2 cell line demonstrated a similar 1.7-kb band

MAGE XP-1	GAGTGTTCGCAACTGGGCCTGGCATGTT	27
MAGE Xp-1	TCAGCGTGGTGTCCAGCAGTGTCTCCCACTCCTTGTGAAGTCTGAGGTTG	77
MAGE Xp-1	CAAAGGACTGTGATCATATGAAGATCATCCAGGAGTACAACCTCGAAATT	127
MAGE Xp-1	CTCAGAAAACAGGACCTTGATGTGAGAGGAGCAGGTTTCAAGTAAACAAAG	177
MAGE Xp-2	<i>ctgacttcgcgttttga</i>	17
MAGE XP-1	GGCGAGGACCCGAGCGAGCTTAAGGCCAGTGGGGTGCAGCGTCTGGTCAG	227
MAGE Xp-2	ggcgaggaccgagcgagtga.....gggggtgcgcgtctggtcag	60
MAGE Xp-1	CCGAGGGTGAATTCTCAGGACTGGTCGGGAGTCAAGGTGCCACATCTCCT	277
MAGE Xp-2	ccaggggtgtattctcaggactggtcggcagtca.....	94
MAGE XP-1	GCCTTTCTGCTCACTTTCCTGCCTGTTTTGCCTGACCACAGCCATCATGC	327
MAGE Xp-2agccatcatgc	105
MAGE Xp-1	CTCGGGGTCAGAAGAGTAAGCTCCGTGCTCGTGAGAAACGCCGAAGCGC	377
MAGE Xp-2	ctcgtggtcagaagagtaagctccgtgcccgtgagaaacgccgaaggcc	155
MAGE XP-1	CGAGAGGAGACCCAGGGTCTCAAGGTTCTGTCACGCCACTGCAGCAGAGAA	427
mage xp-2	cgagatgagacccggggtctcaatgttccctcaggtcactgaagcagagga	205
MAGE XP-1	AGAGGAGTGCCCTCC...TCCTCTCTGTTTATAGGGGATACTCCACAA	474
mage xp-2	agaagaggccccctgctgttccctctctgtttctgggggtgctgcttcaa	255
MAGE Xp-1	GCTCCCCCTGCTGCTGGCATTCCCCAGAAGCCTCAGGGAGCTCCACCCACC	524
MAGE Xp-2	gctctcctgctgctggcattccccagaagcctcagagagcccaaccact	305
MAGE Xp-1	ACCACTGCT...GCTGCAGCTGTGTATGTACCGAATCTGACGAAGGTGC	571
MAGE Xp-2	gccgctgctgcagctgcgggtgttccatccacaaatctaaaaaggtgc	355
MAGE Xp-1	CAAATGC...CAAGGTGAGGAAAAATGCAAGTTTCTCCAGGCCACAACAT	618
MAGE Xp-2	caagagccaccaaggtgagaaaaatgcaagttctcccaggcccaacat	405
MAGE Xp-1	CCACTGAGAGCTCAGTCAAAGATCTGTAGCCTGGGAGGCAGGAATGCTG	668
MAGE Xp-2	ctactagagcccaagcgaagatcctctaaccaggaagtcagggtcgttg	455
MAGE Xp-1	ATGCAC TTCATTCTACGTAAAGTATAAAATGAGAGAGCCCATATGAAGGC	718
MAGE Xp-2	gtgcagttcctgtgtacaagtataaaataaaaagttccgttaaaagggt	505
MAGE Xp-1	AGATATGCTGAAGGTTGTTGATGAAAAGTACAAGGATCACTTCACTGAGA	768
MAGE Xp-2	agaaatgctgaaaattgttgcaaaagggttcaggagcacttcctgaga	555

FIG. 2. Nucleotide sequence alignment of human MAGE Xp-1 and MAGE Xp-2. The MAGE Xp-1 nucleotides are represented by capital letters. The MAGE Xp-2 nucleotides are represented by small letters. Nucleotides that differ are in bold italics.

(Fig. 6). Message was not detected in LCLs or a monkey kidney cell line. The MAGE Xp-2 origin of the RT-PCR product from the Hep-2 control was confirmed by sequencing. No expression of RT-PCR product was observed for MAGE Xp-1 using the same cDNA from the HEp-2 cell line as above and a specific reverse primer from the 3' untranslated region of exon 4 (data not shown).

Because of the strong homology within the MAGE gene family, and particularly the similarity in sequence between MAGE Xp-2 and MAGE Xp-1, we tested the expression of MAGE Xp-2 in breast cancer and melanoma cell lines using RT-PCR; the forward primer was LQ-1F from a nonconserved segment of MAGE Xp-2 (Table 1). The cDNA from one breast carcinoma cell line, MDA-157 (ATCC HTB-24) was

MAGE Xp-1	TCCTCAATGGAGCCTCTCGCCGCTTGGAGCTCGTCTTTGGCCTTGATTTG	818
MAGE Xp-2	tcctcaagaaagcctctgagggcctcagtggtgtctttggccttgagotg	605
MAGE Xp-1	AAGGAAGACAACCCCTAGTAGCCACACCTACACCCTCGTCAGTAAGCTAAA	868
MAGE Xp-2	aataaagtcaaccccacggccacacttacaccttcacgcacaaggtaga	655
MAGE Xp-1	CCTCACCAATGATGGAAACCTGAGCAATGATTGGGACTTTCCCAGGAATG	918
MAGE Xp-2	cctcactgatgaggaatccctgctcagttcctgggactttcccaggagaa	705
MAGE Xp-1	GGCTTCTGATGCCTCTCCTGGGTGTGATCTTCTTAAAGGGCAACTCTGCC	968
MAGE Xp-2	agcttctgatgcctctcctgggtgtgatcttcttaaaaggcaactcagct	755
MAGE Xp-1	ACCGAGGAAGAGATCTGGAAATTCATGAATGTGTTGGGAGCCTATGATGG	1018
MAGE Xp-2	actgaggaagagatctgggaattcctgaatatgttgggagtcctatgatgg	805
MAGE Xp-1	AGAGGAGCACTTAATCTATGGGGAACCCGTAAGTTCATCACCCAAGATC	1068
MAGE Xp-2	agaggagcactcagtccttgggggaacctgggaagctcatcaccaaagatc	855
MAGE Xp-1	TGGTGCAGGAAAAATATCTGAAGTACGAGCAGGTGCCCAACAGTGATCCC	1118
MAGE Xp-2	tgggtgcaggaaaaatatctggagtagcagcaggtgccagcagtgatccc	905
MAGE Xp-1	CCACGCTATCAATTCTATGGGGTCCGAGAGCCTATGCTGAAACCACCAA	1168
MAGE Xp-2	ccacgcttcaattcctgtgggggtccgagagcctatgctgaaaccagcaa	955
MAGE Xp-1	GATGAAAGTCCTCGAGTTTTGGCCAAGATGAATGGTGCCACTCCCCGTG	1218
MAGE Xp-2	gatgaaagtccctggagtttttggccaaggtaaatgggtaccacccccgtg	1005
MAGE Xp-1	ACTTCCCATCCCATTATGAAGAGGCTTTGAGAGATGAGGAAGAGAGAGCC	1268
MAGE Xp-2	ccttcccaacccattacgaagaagctttgaagat...gaagagaaagcc	1052
MAGE Xp-1	CAAGTCCGATCCAGTGTAGAGCCAGGCGTCGCACTACTGCCACGACTTT	1318
MAGE Xp-2	ggagtcctgagccagagttgttagccaggccttgcactactgccatagccaa	1102
MAGE Xp-1	TAGAGCGCGTTCTAGAGCCCCATTGAGCAGGTCTCCACCCCATGTGAG	1368
MAGE Xp-2	tcaatc...tcccaaacccaaagtttaacctgctgttctcaccctcaatgag	1149
MAGE Xp-1	AACTCAGGCAGATTGTTCACTTTGTTTGTGGCAAGATGCC...AACC	1414
MAGE Xp-2	gtcttaggcagattcttactttgttaatt...caaaaggcctgttaacc	1195
MAGE Xp-1	TTTTGAAGTAGTGAGCAGCCAAGATATGGCTAGAGAGATCATCATATATA	1464
MAGE Xp-2	tt.....	1197
MAGE Xp-1	TCTCCTTTGTGTTCTGTAAACATTAGTATCTT.TCAAGTGTTTTCTT	1513
MAGE Xp-2tggtcttggtatgcatgaataacttggtgactttttttttt	1238

FIG. 2—Continued

strongly positive while three other breast carcinoma cell lines, MDA-231, T47-D, and ZR75-1, were negative (Fig. 7). MDA-157 is a medullary carcinoma cell line, while the three other breast carcinoma cell lines that were negative are of ductal origin.

MAGE Xp-2 was strongly expressed in two (M229 and UACC 903) of seven melanoma cell lines and negative in five others (Fig. 8). This pattern did not correlate with the expression patterns of these seven

cell lines for MAGE-1, -2, -3, -4 and MART-1/Melan-A (10; L. Butterfield, personal communication).

DISCUSSION

We have isolated a gene in the MAGE family, MAGE Xp-2, and extended its molecular analysis. MAGE Xp-2 was isolated from a cDNA expression library derived from the human epithelial carcinoma

MAGE Xp-1 T.....TAATAGAATGTTTATTATTAGAGTTGGGATCTAT 1546
 MAGE Xp-2 tctcttttttcaactagtgtttcaacaggtttatttagattcagaatgtaa 1288

 MAGE Xp-1 GTCTATGAGCGACATGGATCACACATTATTGGTG.CTGCCAGCTTTAAG 1595
 MAGE Xp-2 atttacaagtatatagatcacccgttatttgctgttttcagggaagct 1338

 MAGE Xp-1 CATAAGAGTTTGTATATT.....CTATATTTTCAAATCCTTG 1633
 MAGE Xp-2 agaaagtgttttggttttttgagtgaacaacttattaataaaaatcctta 1388

 MAGE Xp-1 AATCTTTTTTGGGTGAAGAAGAAGAAAGCATAGCTTTAGAATAGAGATT 1683
 MAGE Xp-2 aatctcttttgtaaccagggaagaagaaatgtggcattagaatagaata 1438

 MAGE Xp-1 TTCTCAGAAATGTGTGAAGAACCTCACACAACATAATTGGAGTCTTAAAA 1733
 MAGE Xp-2 tcttgggaaatgtg.aagaacccatagtgaataattgggatcagaagc 1487

 MAGE Xp-1 TAGAGGAAGAGTAAGCAAAGCATGTCAAGTTTTTGTCTTCTGCATTTCAGT 1783
 MAGE Xp-2 cagagg.....ggtaaaaagtgggtcaattcttggtttacttcatttaatt 1530

 MAGE Xp-1 TTTGTTTTTGTAAAATCCAAAGATACATACCTGGTTGTTTTTAGCCTTTT 1833
 MAGE Xp-2 ctttctttt.....cataaagatacatacctggatttgtttatgttatt 1574

 MAGE Xp-1 CAAGAATG.CAGATAAAATAAATAGTAATAAATT 1866
 MAGE Xp-2 caagaatgtgtgagaaattaaaccatagttagtt 1608

FIG. 2—Continued

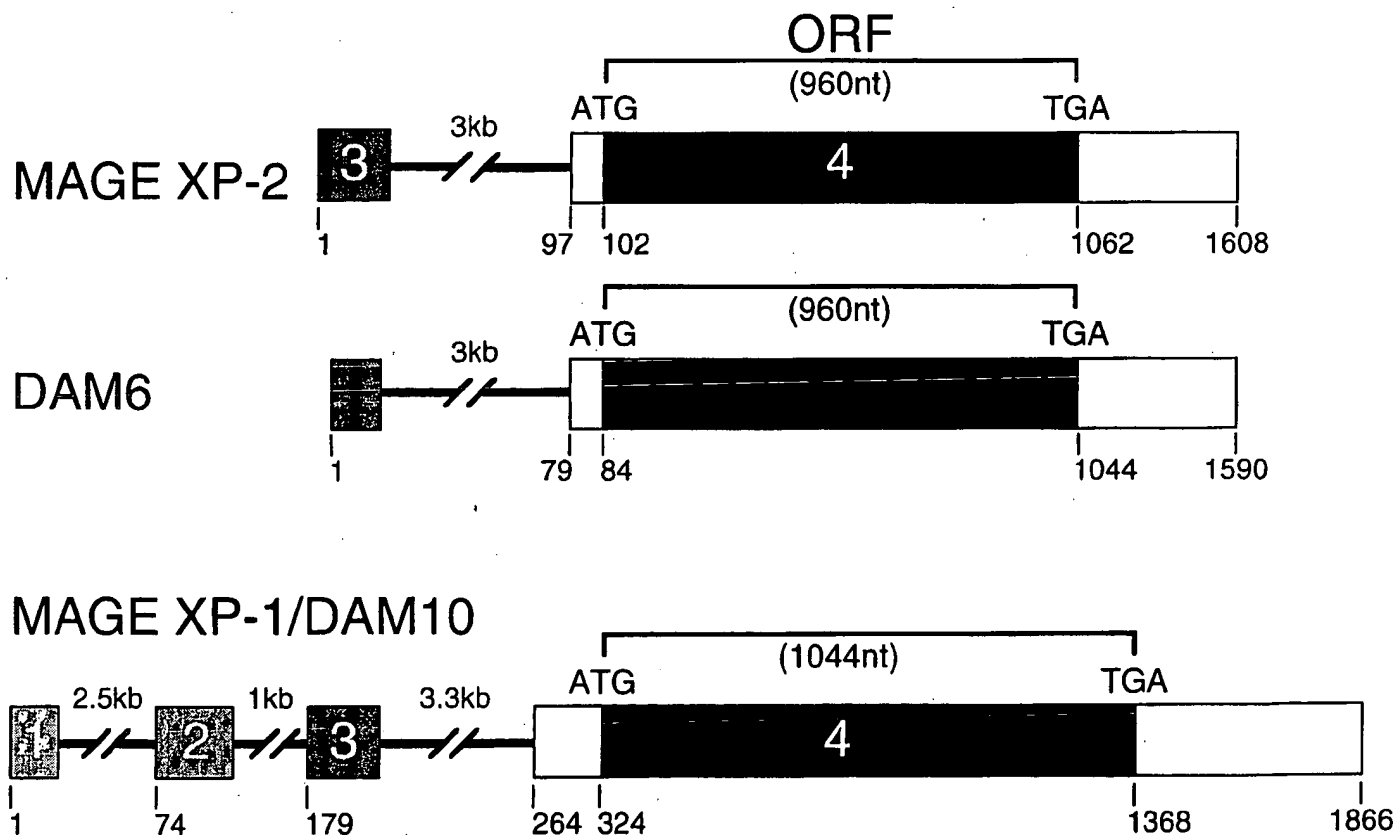


FIG. 3. Structure of MAGE Xp-2, DAM 6, and MAGE Xp-1/DAM 10. The exons are expressed as boxes. The open reading frame (ORF) is in exon 4 for all three genes. Both MAGE Xp-2 and DAM 6 have an ORF of 960 bp that encodes a predicted protein of 319 amino acids. MAGE Xp-2 and MAGE Xp-1 sequences diverge at the 5' end of exon 3 (see also Fig. 2).

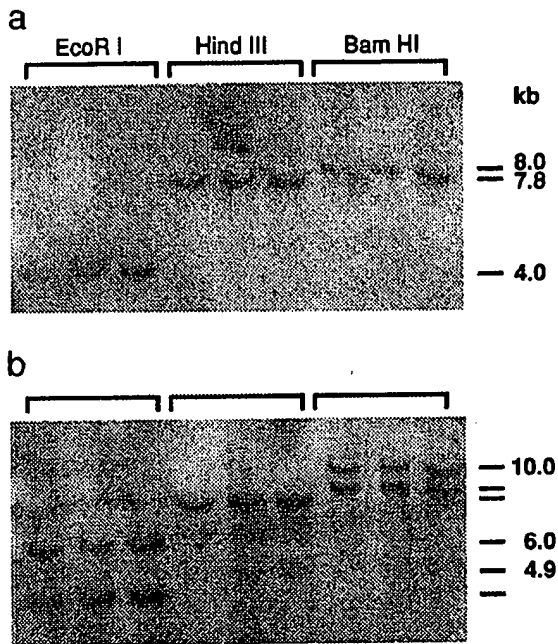


FIG. 4. Southern blot using DNA from normal PBM. DNA was digested with *EcoRI*, *HindIII*, and *BamHI* and was first probed with PCR-amplified MAGE Xp-1 product. (a) Three bands were observed: *EcoRI* 4.0 kb, *HindIII* 7.8 kb, and *BamHI* 8.0 kb. (b) Without strip washing, the blot was probed again with a PCR-amplified MAGE Xp-2 product. Three new bands were observed: *EcoRI* 6.0 kb, *HindIII* 4.9 kb (weak), and *BamHI* 10.0 kb.

cell line, HEp-2. The HEp-2 cell line was chosen because this cell line is routinely used in diagnostic laboratories to detect ANA in patients with SLE. We screened the expression library with two sera from patients with high titer ANA and lupus nephritis.

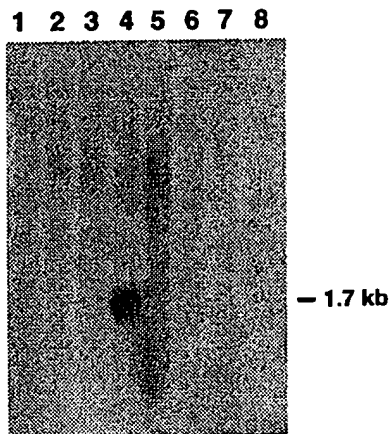


FIG. 5. Expression pattern of MAGE Xp-2 by Northern blot analysis. A commercially prepared Northern blot (CLONTECH) of normal tissue was probed with a MAGE Xp-2-specific PCR product. MAGE Xp-2 expression was detected only in testes (lane 4). Lanes: 1, spleen; 2, thymus; 3, prostate; 4, testes; 5, ovary; 6, small intestine; 7, colon (mucosal lining); 8, peripheral blood leukocytes.

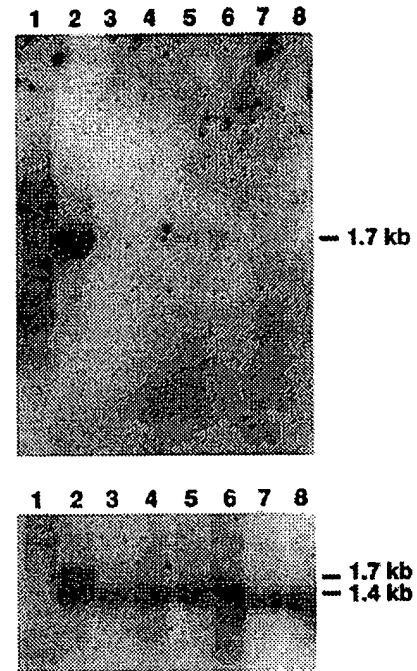


FIG. 6. Expression of MAGE Xp-2 in the HEp-2 cell line by Northern blot analysis. (Top) Total RNA was prepared from various cell lines including human epithelial carcinoma (HEp-2), monkey kidney, and EBV-transformed LCLs; a blot was probed with MAGE Xp-2 (clone 7) insert. Only HEp-2 was positive. (Bottom) A G3PDH cDNA probe was used as a control. Lanes: 1, RNA size marker; 2, HEp-2 (epithelium); 3, COS-7 (monkey kidney); 4, CHOC-12 (SLE); 5, NAT-1 (SLE); 6, NAT-9 (normal); 7, CHOC-6 (normal); 8, SYO (A-T).

One of the transcripts recovered showed approximately 66% homology with a previously described MAGE gene, MAGE Xp-1 (6). Since MAGE Xp-2 was also located in the MAGE Xp region, but was distinct from MAGE Xp-1, we termed this gene MAGE Xp-2. Subsequently, we found that the ORF of MAGE Xp-2 was almost identical to another MAGE Xp sequence, DAM 6 (7).

We designed PCR primers that clearly distinguished MAGE Xp-1 from that of MAGE Xp-2 and then further analyzed the tissue expression of MAGE Xp-2. Initial reports for MAGE Xp-1 (6) did not demonstrate its expression in cancer cell lines; however, subsequent studies by Dubovic *et al.* (7) on DAM 10 (i.e., MAGE Xp-1) and DAM 6 (i.e., MAGE Xp-2) did show expression in lung cancer including squamous cell carcinomas, adenocarcinomas, and undifferentiated carcinomas. Our studies show MAGE Xp-2 expression in the epithelial carcinoma cell line, HEp-2, as well as in several breast cancer and melanoma cell lines that were also of epithelial origin. The PCR products were confirmed by sequencing.

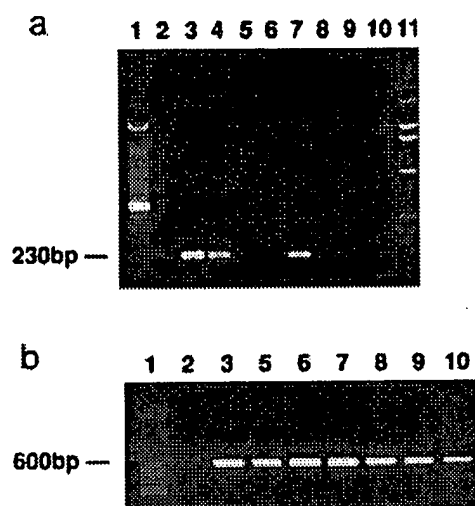


FIG. 7. RT-PCR for MAGE Xp-2. (a) Gene expression was detected by the expected 230-bp product using total RNA and mRNA derived from the HEP-2 cell line (lane 3 and 4, respectively), as well as from one of four breast carcinoma cell lines; lane 7, MDA-157. No expression was found in two melanoma cell lines; this result is repeated in Fig. 8. (b) The cDNAs used above were all positive for the expected 600-bp RT-PCR product when amplified with primers for exons 51–54 of the ATM (ataxia telangiectasia mutated) gene. Lanes: 1, size marker; 2, negative control; 3, HEP-2 (epithelium); 4, Hep-2 (mRNA); 5, M24 (melanoma); 6, M202 (melanoma); 7, MDA-157 (breast); 8, MDA-231 (breast); 9, T47-D (breast); 10, ZR75-1 (breast); 11, size marker.

Immunization with MAGE-encoded antigens may prove to be a powerful tool in melanoma therapy. Understanding the patterns of MAGE gene expression in normal and cancer tissues may speed progress in this important endeavor. Human melanoma cells process the MAGE-1 gene product and present the processed nonapeptide with their major histocompatibility complex class I molecules stimulating CTLs. In a recent study, autologous antigen presenting cells (APCs) pulsed with the synthetic nonapeptide were found to be capable of inducing autologous melanoma-reactive and nonapeptide-specific CTLs (11). When patients were immunized with the cultured autologous APCs pulsed with the synthetic nonapeptide, CTLs were found both at the immunization site and at distant metastatic sites (11). MAGE genes are expressed on a variety of tumor cell lines, but only in testes and placenta of normal tissue. Recent data show that the expression of the MAGE genes in testis is restricted to germ-line cells (12). This might be expected to cause a major iatrogenic reaction when using melanoma vaccine therapy. However, since male germ-line cells lack expression of the major HLA molecules, they should not express immunogenic MAGE antigens. It also follows then that CTLs specific for MAGE peptides

would not be eliminated or functionally inactivated during normal development as would be expected to occur with CTLs specific for self peptides expressed in other normal tissues.

The finding of MAGE Xp-2 expression in a medullary breast carcinoma is of note. This nonductal breast carcinoma tends to grow as a solid mass surrounded by a lymphoreticular infiltrate. The prognosis is better for medullary breast carcinomas than for ductal breast carcinomas. As in melanoma, it is possible that breast carcinomas which express MAGE genes elicit an immune response that may control tumor growth and metastases.

MAGE genes have several interesting characteristics that we speculate may explain the isolation of MAGE Xp-2 with sera from patients with SLE. A recent study has shown that normal cells of the skin basal layer transiently express MAGE-1 during wound healing (13). Subsequent studies by De Smet *et al.* (14) and Shichijo *et al.* (15) have demonstrated that this gene can be activated by demethylation. The promoter region of MAGE-1 contains several

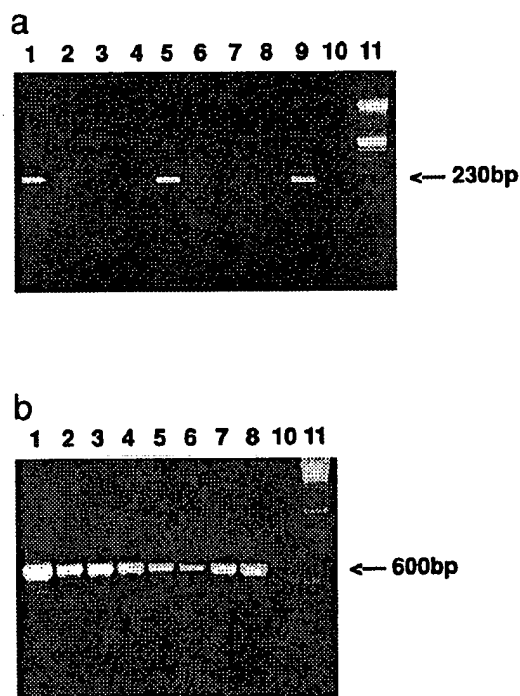


FIG. 8. RT-PCR for MAGE Xp-2. (a) Using total RNA from seven melanoma cell lines, the expected 230-bp product was detected in two (lanes 5, M229, and 9, UACC 903). Total RNA from two different sources of M202 was handled independently; both samples were negative. (b) The cDNAs used above were all positive for the expected 600-bp RT-PCR product when amplified with primers for exons 51–54 of the ATM (ataxia telangiectasia mutated) gene. Lanes: 1, HEP-2 (epithelium); 2, M201; 3, M202; 4, M207; 5, M229; 6, M25IT; 7, M24; 8, M202; 9, UACC 903; 10, negative control; 11, size marker.

positive regulatory elements between positions -792 and +47 which drive 90% of the promoter activity (14). Two key promoter elements contain identical, but inverted, *Ets* transcription factor binding sites. De Smet *et al.* (14) have further shown that the *Ets* binding site contains a CpG dinucleotide that when methylated inhibits binding of the transcription factors (16,17). Treatment with the demethylating agent, 5-aza-2'-deoxyxycytidine, resulted in activation of the MAGE-1 gene in fibroblasts (13) and MAGE-1, -2, -3, and -6 in normal and malignant lymphoid cells (15). If patients with SLE express MAGE genes after tissue injury (i.e., viral, UV light, drug sensitivity, chemical toxicity), this might activate not only a cytotoxic T cell response but also a humoral component producing MAGE-related autoantibodies. The fact that immune presentation of MAGE-1 is HLA-dependent may provide a mechanism to explain the HLA Class I association with SLE (18).

We are currently studying MAGE Xp-2 expression in tissues from patients with SLE and other inflammatory diseases. We are also developing an experimental model for screening SLE sera for antibodies to MAGE Xp-2 protein. Taken together, the data reported herein suggest new potential roles for MAGE gene products in autoimmunity, breast cancer, melanoma, tissue repair, and inflammation.

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